Site-directed Mutagenesis of Tetraheme Cytochrome c₃

MODIFICATION OF OXIDOREDUCTION POTENTIALS AFTERHEME AXIAL LIGAND REPLACEMENT*

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The nature of the axial ligands of a heme group is an important factor in maintaining the oxidation-reduction potential of a c-type cytochrome. Cytochrome c₃ from Desulfovibrio vulgaris Hildenborough contains four bis-histidinyl coordinated hemes with low oxidation-reduction potentials. Site-directed mutagenesis was used to generate a mutant in which histidine 70, the sixth axial ligand of heme 4, has been replaced by a methionine. The mutant protein was expressed in Desulfovibrio desulfuricans G200 at a level similar to the wild type cytochrome. A model for the three-dimensional structure of D. vulgaris Hildenborough cytochrome c₃ was generated on the basis of the crystal structure of D. vulgaris Miyazaki cytochrome c₃ in order to investigate the effects of the H70M mutation. The model, together with NMR data, suggested that methionine 70 has effectively replaced histidine 70 as the sixth axial ligand of heme 4 without significant alteration of the structure. A large increase of at least 200 mV of one of the four oxidation-reduction potentials was observed by electrochemistry and is interpreted in terms of structure/potential relationships.

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The nature of the axial ligands of a heme group is an important factor in maintaining the oxidation-reduction potential of a c-type cytochrome. Cytochrome c₃, isolated only from the sulfate reducing bacteria, is a good candidate for this purpose. The cytochrome c₃ superfamily is characterized by the presence of four hemes of low oxidation-reduction potential, bound to the polypeptide moiety through two thioether linkages with cysteine side chains (5). The low oxidation-reduction potentials (around −300 mV) confer an important function to this class of cytochromes in the anaerobic respiration of sulfate (6). The four heme oxidation-reduction potentials differ from each other by up to 235 mV (2). The iron atom of each heme group is coordinated by two histidine side chains as in b-type cytochrome (3), rather than histidine and methionine as in most monoheme c-type cytochromes.

The three-dimensional structures of both Desulfovibrio desulfuricans Norway and Desulfovibrio vulgaris Miyazaki cytochromes c₃ have been solved at 2.5-Å and 1.8-Å resolution, respectively (7, 8), and, very recently, the structure of D. vulgaris Hildenborough cytochrome c₂ has been determined at 2-Å resolution (9). It appears that the folding pattern of these cytochromes is very similar, the heme core geometry being highly conserved in the structures. In each structure, the heme groups are located in different protein environments.

Cytochrome c₂ has been described as an electron carrier between hydrogenase and ferredoxin (10). Biophysical studies have shown specific in vitro interactions between cytochrome c₂ and ferredoxin (11). Computer graphics modelling and cross-linking experiments have allowed the identification of heme 4 as the reactive heme in D. desulfuricans Norway cytochrome c₂-ferredoxin I complex (12, 13). This complex formation is followed by a bidirectional electron transfer between the heme and the (4Fe-4S) cluster (14). Heme 4 of D. vulgaris cytochrome c₂ is thought to interact with both flavodoxin and rubredoxin (15). Based on chemical modification techniques (16) and EPR (17) studies, contradictory oxidation-reduction potential assignments to this heme have been proposed. Another powerful method, to clarify this assignment and to specify the structure/oxidation-reduction potential relationships, is site-directed mutagenesis.

The gene of D. vulgaris Hildenborough cytochrome c₃ has been cloned and sequenced (18). Recently, functional expression of this cytochrome has been obtained in D. desulfuricans G200 after conjugal gene transfer from Escherichia coli (19).

As the D. vulgaris Miyazaki and D. vulgaris Hildenborough...
cytochrome c₃ sequences share 88% of sequence identity (5), a model of the *D. vulgaris* Hildenborough cytochrome c₃ structure is presented in this paper. Availability of both this model and a method for functional expression makes this cytochrome an interesting system for site-directed mutagenesis and biophysical experiments. Our aim is to complete the assignment of the large mid-chain-reduction potentials and elucidate their function. The present study establishes the effects, in terms of structure and oxidation-reduction potential changes, of replacement of histidine 70, the sixth axial ligand of heme 4, by a methionine residue.

**MATERIALS AND METHODS**

**Strains, Vectors, and Growth Conditions**—The bacterial strains and plasmids used in this study are described in Table I. Growth of *E. coli* strains was carried out in TY medium (20), supplemented with the appropriate antibiotic. Liquid cultures were shaken at 250 r.p.m. in a New Brunswick G 25 shaker at 37 °C. *E. coli* DH5α and *D. desulfuricans* G200, used for conjugational transfer of broad-host-range vectors, were cultured as previously described (19). Large scale growth of *D. desulfuricans* exconjugants (*D. desulfuricans* G200 (pJRD800-1) and *D. desulfuricans* G200 (pJRD800M70)) was in Postgate C medium (21) supplemented with 0.2 mM kanamycin.

**Biochemical Reagents**—All enzymes used for cloning and sequencing were obtained from Pharmacia LKB Biotechnology Inc. The radiochemical [α-32P]SdATP (400 Ci mmol⁻¹) was purchased from Amersham and was used for deoxynucleotide sequencing. A mutagenic oligonucleotide was purchased from the Regional DNA Synthesis Laboratory of the University of Calgary.

**Site-directed Mutagenesis**—Plasmid p800 (Table I) served as the source for the cytochrome c₃ (cyc) gene from *D. vulgaris* Hildenborough. Purified p800 DNA was digested with restriction enzymes EcoRI and HindIII, and the 640-base pair EcoRI-HindIII fragment, which contained the cyc gene, was isolated by agarose gel electrophoresis in 40 mM Tris acetate buffer (pH 8.0) containing 20 mM EDTA. The isolated fragment was ligated to the replicative form of M13 mp8, which had been digested with the same two enzymes, giving M13 mp800. The mutagenic oligonucleotide P52 (5' TTTCAAGATCC- TGACAT) was designed to change the CAT codon at nucleotides 427–429, encoding histidine 70, into ATG. The method of Kunkel (22), in which uracil is incorporated into the single-stranded DNA template, was used to enrich mutant copies of the cloned gene.

The single-stranded M13 mp800 DNA template, which contains uracil, was isolated from the M13 phage grown on the *E. coli* RE2032 strain (23). Mutagenesis was carried out by in vitro hybridization of mutagenic primer to the DNA template, in a 10:1 molar ratio followed by extension with the Klenow fragment of DNA polymerase I and ligation with T4 DNA ligase. Competent *E. coli* DH5α was transformed with the mutagenesis reaction mixture, and single-stranded M13 DNAs were sequenced by the dideoxy chain termination method (23) to screen for the desired base replacements. The replicative form of M13 DNA, carrying the desired mutation, was digested with both EcoRI and HindIII. The 640-base pair gel-isolated fragment was ligated to pJRD215, previously digested with the same two enzymes, to give pJRD800M70. This recombinant plasmid was transformed into *E. coli* DH5α and subsequently transferred to *D. desulfuricans* G200 as previously described (19).

**Purification of Both Wild Type and H70M Cytochromes c₃ Overexpressed in D. desulfuricans G200**—The purification procedure of wild type cytochrome c₃ was that of Voodoow et al. (19). For the purification of H70M cytochrome c₃, 340 g of cells (wet weight) of *D. desulfuricans* G200 (pJRD800M70), obtained from a 300-liter fermentation, were harvested as previously described (19). The mutant H70M cytochrome c₃ was isolated from the cell paste with a procedure essentially the same as for the wild type cytochrome (19).

**Acrylamide Gel Electrophoresis—SDS-PAGE**— was performed according to Laemmli (24) using 13% (w/v) polyacrylamide slab gels.

**Amino Acid Analysis and Protein Sequencing**—Amino acid analyses were carried out on a Beckman amino acid analyzer (system 6300). Protein samples were hydrolyzed in 6 M HCl at 110 °C for 20 h in evacuated sealed tubes. The NH₂-terminal sequence determinations were performed on an Applied Biosystems A 470 Gas Phase Sequenator. Quantitative determination of phenylthiohydantoin derivatives was done by high pressure liquid chromatography (Waters Associates, Inc.) monitored by a data and chromatography control station (Waters 840).

**Analysis for Home Content**—The number of home units was determined with the pyridine ferrohemochromogen test (25).

**Spectrophotometric Studies**—Visible and ultraviolet absorption spectra were determined with a Beckman DU 7500 spectrophotometer. Molar extinction coefficients at the absorption maximum (553 nm) were obtained from these spectra using protein concentrations derived from amino acid analysis.

**NMR Spectroscopy**—For NMR experiments, the protein samples (18° m) were prepared by H₂O exchange by successive lyophilizations in 0.1 M phosphate buffer (pH 7.6). Full reduction of the samples was obtained by addition of two electron equivalents of diosodium dithionite in 0.1 M phosphate buffer at pH 7.6, under nitrogen atmosphere. H NMR spectra were recorded in the Fourier mode on a Bruker AM200 spectrometer, at 35 °C. The water line was irradiated during these experiments (15 liters). Chemical shifts are in parts per million from internal tetramethylsilane.

**Computer Graphics Modelling**—The construction of the three-dimensional models of both *D. vulgaris* Hildenborough wild type and H70M cytochromes c₃ was based on the chemical sequence and the three-dimensional structure of the homologous cytochrome c₃ from *D. vulgaris* Miyazaki (8). Atomic coordinates of the latter were obtained from the Protein Data Bank, and sequence modifications (14 of 107 residues) were performed with the TURBO-FRODO program (36) implemented on a Silicon Graphics 4D/25 color display workstation.

**Electrochemistry**—Cyclic voltammetry and differential pulse voltammetry (with a pulse amplitude of 25 mV) were carried out using an EG&G 273 potentiostat modulated by an IBM XT microcomputer with M270 software. For alternating current voltammetry, the potentials were performed on an Applied Biosystems A470 Gas Phase Sequenator. Quantitative determination of phenylthiohydantoin derivatives was done by high pressure liquid chromatography (Waters Associates, Inc.) monitored by a data and chromatography control station (Waters 840).

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was used throughout. The pyrolitic graphite electrode was constructed from 4-mm diameter graphite rods (Le Carbone Lorraine, Paris) cut with the disk face parallel to the basal plane (a-b) housed in epoxy sheaths. Prior to each experiment, the PG electrode was polished with an ultrafine emery paper, then with 0.05-μm alumina slurry, and then washed with distilled water. The freshly polished gold (1-mm diameter) electrode was modified by dipping it into 1 mM bis(4-pyridyl) disulfide (aldrithiol) solution for 2 min. The supporting buffer at pH 7.6. Oxygen was purged from solution by gently bubbling with high purity grade nitrogen. Measurements were performed at 28 °C.

RESULTS

Biochemical Characterization of the Mutant Cytochrome c₃—The mutant H70M cytochrome c₃ was obtained from the periplasmic protein fraction. The purification was identical with that previously described for wild type cytochrome c₃ (19), suggesting that no changes in overall physical properties were induced by the H70M replacement. The mutant protein was found to be pure by SDS-PAGE and had a purity coefficient C \[\frac{[A_{570\text{red}}] - [A_{280\text{ox}}]}{[A_{280\text{ox}}]}\] of 3.0. The yield (60 mg/340 g of cells) was similar as obtained for wild type cytochrome c₃ (19). Biophysical characteristics of both wild type and H70M cytochromes c₃ are shown in Table II.

The spectral properties of the mutant cytochrome c₃ were the same as those of the wild type protein except in the 695 nm region where a weak band appeared. This band is characteristic of a histidine-methionine coordination of the heme iron generally found in c-type cytochromes (27). The detection is harder in the case of H70M cytochrome c₃ than in monoheme cytochromes because it is overlaid by the absorbance of the three bis-histidinyl coordinated hemes. The molar extinction coefficient at 553 nm in the fully reduced state was determined to be 115,000 M⁻¹ cm⁻¹ as in the case of wild type cytochrome c₃.

The amino acid composition of the mutant cytochrome c₃ was in complete agreement with that of the wild type D. vulgaris Hildenborough cytochrome c₃ except for the histidine and methionine contents. The NH2-terminal sequence of the mutant cytochrome was found to be identical with that previously described for wild type cytochrome c₃ (29). The position of this methyl line in ferricytochrome c are generally found at 1 ppm instead of at 8 ppm in most of the proteins (29) eliminates assignment of this line to histidine ring protons. Moreover, the line observed at -3.2 ppm is similar to the one in methylimethionine line found in reduced monoheme cytochromes c NMR spectra (Table III) (29). The position of this methyl line in ferricytochrome c depends on the source of the cytochrome. The high field resonances of H70M cytochrome c₃ have the same pattern as those of cytochrome c₃ (29), the monoheme cytochrome isolated from the same bacterium. The chemical shift of the high field resonance at -7.15 ppm in the oxidized state (Fig. 3a) and -3.2 ppm in the reduced state (Fig. 3b), when compared with the wild type. The fact that ring proton lines of the histidine axial ligand in cytochrome c are generally found at 1 ppm instead of at 8 ppm in most of the proteins (29) eliminates assignment of this line to histidine ring protons. Moreover, the line observed at -3.2 ppm is similar to the one in methylimethionine line found in reduced monoheme cytochromes c NMR spectra (Table III) (29). The position of this methyl line in ferricytochrome c depends on the source of the cytochrome. The high field resonances of H70M cytochrome c₃ have the same pattern as those of cytochrome c₃ (29), the monoheme cytochrome isolated from the same bacterium. The chemical shift of the high field resonance at -7.15 ppm in the oxidized state is temperature-dependent, indicating that it belongs to a methyl group of a heme axial ligand. This resonance disappears after addition of large excess of KCN to the solution; reduction with sodium dithionite allows the recovery of the resonance at -3.2 ppm as previously described by Wüthrich (29) (data not shown). These observations provide strong evidence that the sixth axial ligand of heme 4 is a methionine residue in both the ferric and the ferrous oxidation states of the mutant protein.

Electrochemistry—Typical 'CV and 'DPV curves for wild type and H70M mutant cytochrome c₃ solutions are given in Fig. 4, a and b, respectively. For wild type cytochrome c₃, a single cathodic wave at -560 mV and a single anodic wave at -500 mV are observed in Fig. 4a. A single DPV peak is also detected at \(E_p = -530\) mV. These results confirm previous

<table>
<thead>
<tr>
<th>D. vulgaris Hildenborough cytochrome c₃</th>
<th>pI</th>
<th>(\varepsilon_{562\text{red}})</th>
<th>Heme content per molecule</th>
<th>Molecular weight</th>
<th>Redox potentials (0.1 M phosphate buffer (pH 7.6)) (mV vs. NHE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.5</td>
<td>115,000</td>
<td>3.5 (4)</td>
<td>16,029</td>
<td>-280, -320, -350, -380 (±10 mV)</td>
</tr>
<tr>
<td>H70M</td>
<td>10.5</td>
<td>115,000</td>
<td>3.5 (4)</td>
<td>16,024</td>
<td>-80, -260, -320, -390 (±15 mV)</td>
</tr>
</tbody>
</table>
data on *D. vulgaris* cytochrome *c₃* (30), indicating that the four oxidation-reduction potentials have closely similar values (Table II). In contrast, the electrochemical behavior of H70M mutant cytochrome *c₃* is significantly different from that of the wild type protein (Fig. 4b). Two reduction-reoxidation couples are detected by cyclic voltammetry at −320/−220 mV (couple 1) and −570/−470 mV (couple 2), and the DPV curve exhibits two peaks at −270 and −520 mV. From the shapes and peak heights of both CV and DPV curves, it can be assumed that couple 1 corresponds to the reduction-reoxidation of a single heme with a peak separation of around 100 mV and couple 2 to the reduction-reoxidation of the three other hemes.

Of special interest are the results obtained by using the modified aldrithiol-gold electrode. No peak is observed on the ¹ACV curve for wild type cytochrome *c₃* in Fig. 5 (curve a). In contrast, a peak corresponding to a relatively fast electrochemical process appears at −270/−260 mV on forward and backward sweeps for the mutant protein solution (curve b). This peak is not detected at the unmodified polished gold electrode (curve c). Similar results are obtained from CV and DPV experiments. In previous works it has been shown that the modifying gold electrode surface allows direct investigations of the electrochemical behavior of some hemoproteins such as cytochrome *c* (31) and other bacterial c-type cytochromes (32) which have relatively high oxidation-reduction potentials. It is striking to note that all hemoproteins, which give an electrochemical response at the modified gold electrode, possess methionine/histidine as axial iron ligands. As the four hemes are correctly inserted in the well known three-dimensional structures of cytochromes *c*, this can be related to the presence of the axial methionine ligand.

The oxidation-reduction potentials of wild type and mutant cytochrome *c₃* were determined by using the equations established for DPV curves at solid electrodes (33) and the theoretical treatment previously described (34). The results show that one oxidation-reduction potential has been strongly affected by the mutation (Table II).

**DISCUSSION**

As an additional strategy to probe metalloprotein structure and function, site-directed mutagenesis is useful for preparing mutant proteins that differ from the natural system in the ligation of the metal ions. In the case of cytochromes, Hampsey et al. (35) have stated that replacements in ligation positions would be expected to abolish function, presumably by altering the oxidation-reduction potential of the heme or by changing the protein folding pattern. This was confirmed by experiments on mutant cytochrome *c₁* in the cytochrome *b₁-c₁* complex (36).

We have constructed a mutant of cytochrome *c₃* with a methionine instead of a histidine as the sixth axial ligand of heme 4. This mutant protein was obtained in the same yield as the wild type from *D. desulfuricans* G200 exconjugants and has biochemical and spectrophotometric properties that are identical with the wild type cytochrome. A specific protein, referred as heme lyase, is required for heme insertion into apocytochrome *c₃*, giving a functional enzyme. The mutant cytochrome *c₃* differs from the wild type molecule by only the sixth axial ligand of heme 4. NMR spectroscopy provided strong evidence that methionine 70 replaces histidine as this axial ligand. As the four hemes are correctly inserted in the protein, the heme lyase system can covalently attach the heme groups to the apoprotein even if the iron coordination is different. Computer graphics modelling suggests that the mutation H70M can be accommodated by the protein structure without drastic changes in the folding of the cytochrome.

This substitution induces a large shift of one of the four oxidation-reduction potentials. The following must be kept in mind in interpreting this effect. (i) The H70M mutation could induce conformational changes within the heme core environment. (ii) Gayda et al. (37) and Fan et al. (38) have proposed the existence of interacting midpoint potentials within *D. vulgaris* Miyazaki cytochrome *c₃*. This can be related to the well known three-dimensional structures of cytochromes *c₂* (7, 8) in which, for example, a strong electronic coupling between hemes 3 and 1 is enhanced by the intervening aromatic side chain, Phe-20 (numbering of the *D. vulgaris* Miyazaki sequence), that is strictly conserved in all known cytochrome *c₂* sequences. This and other structural features previously discussed (39) play a critical role in the intramolecular electron transfer within cytochromes *c₂*. Coupling between the heme groups of *D. vulgaris* Miyazaki cytochrome *c₃* can be extrapolated to the Hildenborough structure and, in consequence, the change of one oxidation-reduction potential could influence the three others.

**FIG. 1.** Stereo view of the model of the three-dimensional structure of *D. vulgaris* Hildenborough cytochrome *c₃*, based on the atomic coordinates of *D. vulgaris* Miyazaki cytochrome *c₃*, Substitutions of side chains are shown.
Fig. 2. Model of the heme 4 environment in wild type (a) and H70M (b) cytochromes c₃. Axial ligands of the heme 4 iron are indicated.
The first observation is that the H70M mutation increases one of the oxidation-reduction potentials by at least 200 mV. However, the oxidation-reduction potential values for the wild type protein (−280, −320, −350, and −380 mV) are too similar to determine which one is changed by electrochemical techniques. EPR experiments will provide more information for the assignment of the oxidation-reduction potentials to the hemes in the structure.

The observed change is similar to the decrease of 230 mV obtained after substitution of the sixth axial methionine residue by a histidine in semisynthetic cytochrome c (40). Moreover, a comparable effect was reported by Moore and Williams (41) on synthetic heme derivatives. On the other hand, replacement of the histidine fifth axial ligand by an arginine in cytochrome c does not affect the oxidation-reduction potential but perturbs the rate of electron transfer (42). Our data therefore confirm the importance of the sixth axial ligand in maintaining the heme oxidation-reduction potential.

TABLE III

<table>
<thead>
<tr>
<th>C-type cytochrome species</th>
<th>Methionine axial ligand</th>
<th>Chirality (ox)</th>
<th>Chirality (red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>horse</td>
<td>−24.8 (20 °C)</td>
<td>R</td>
<td>−3.28 (20 °C)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>−3.28 (20 °C)</td>
<td>R</td>
<td>−3.28 (20 °C)</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>−23.6 (35 °C)</td>
<td>R</td>
<td>−3.13 (35 °C)</td>
</tr>
<tr>
<td>R. rubrum</td>
<td>−22.4 (35 °C)</td>
<td>R</td>
<td>−3.20 (35 °C)</td>
</tr>
<tr>
<td>R. gelatinosa</td>
<td>−14.9 (30 °C)</td>
<td>S</td>
<td>−2.84 (30 °C)</td>
</tr>
<tr>
<td>P. mendocina</td>
<td>−9.9 (35 °C)</td>
<td>S</td>
<td>−2.84 (35 °C)</td>
</tr>
<tr>
<td>P. stutzeri</td>
<td>−3.13 (35 °C)</td>
<td>R</td>
<td>−2.84 (35 °C)</td>
</tr>
<tr>
<td>Desulfouibrio</td>
<td>−16.4 (35 °C)</td>
<td>S</td>
<td>−2.84 (35 °C)</td>
</tr>
<tr>
<td>Desulfo vulgaris</td>
<td>−17.6 (25 °C)</td>
<td>S</td>
<td>−2.96 (25 °C)</td>
</tr>
<tr>
<td>Desulfo vulgaris</td>
<td>−15.3 (35 °C)</td>
<td>S</td>
<td>−2.90 (30 °C)</td>
</tr>
<tr>
<td>Desulfo vulgaris</td>
<td>−10.2 (35 °C)</td>
<td>S</td>
<td>−3.02 (30 °C)</td>
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<td>Desulfo vulgaris</td>
<td>−8.7 (35 °C)</td>
<td>R</td>
<td>−3.62 (30 °C)</td>
</tr>
<tr>
<td>Desulfo vulgaris</td>
<td>−9.5 (35 °C)</td>
<td>N*</td>
<td>−3.62 (35 °C)</td>
</tr>
</tbody>
</table>

* ND, not determined.
Site-directed Mutagenesis on Tetraheme Cytochrome c₃

related to the apolarity of heme environment. Various workers have shown that a change in charge and size of an invariant heme contact residue, which does not cause a major change of conformation, affects the heme oxidation-reduction potential (49). Similarly, Caffrey et al. (50) have shown the importance of a conserved hydrogen-bonding network in cytochromes c in determining heme oxidation-reduction potentials.

As shown above, modelling of the D. vulgaris Hildenborough H70M cytochrome c₃ mutation suggests the absence of drastic structural perturbations of the heme 4 environment. However, the mutation disrupts the wild type hydrogen bond between the carbonyl group of Tyr-66 and the Nᵦ of His-70. The H70M substitution could lead to a conformational change of Tyr-66, bringing its hydroxyl group within ~3 Å to the Met-70 sulfur atom. In fact, such a distance is found in tuna cytochrome c and Rhodopseudomonas rubrum cytochrome c₂ (51). The electrostatic interaction between the tyrosine hydroxyl group and the methionine sulfur is known to stabilize the oxidized state and therefore to decrease the oxidation-reduction potential. Although the oxidation-reduction potential change of heme 4 in the H70M cytochrome c₃ is mainly caused by the His/Met substitution, the structural factors discussed above can contribute to this oxidation-reduction potential change. Crystallographic structures at high resolution of both wild type and mutant cytochromes are necessary to further clarify these points.

The functional properties of the H70M cytochrome c₃ are very interesting. Preliminary data indicate that the mutant cytochrome can be reduced by hydrogenase under hydrogen atmosphere (data not shown). Kinetic studies will characterize the electron transfer rate between these oxidation-reduction partners. Moreover, an intramolecular electron exchange at the oxidized state and therefore to decrease the oxidation-reduction potential of both wild type and mutant cytochromes is in progress and will allow a full assignment of the various oxidation-reduction potentials to the four hemes in the three-dimensional structure of the D. vulgaris Hildenborough cytochrome c₃.

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REFERENCES

FIG. 4. Cyclic (left) and differential pulse (right) voltammetry at a pyrolytic graphite electrode of wild type (a) and H70M (b) cytochrome c₃, at 28 µM in 0.1 M phosphate buffer, pH 7.6. Scan rate: 5 mV s⁻¹. Potentials are expressed versus Ag/AgCl (saturated NaCl) reference electrode.

FIG. 5. Alternating current voltammetry at an anodic modification gold electrode of wild type (a) and H70M (b) cytochrome c₃ in 0.1 M phosphate buffer, pH 7.6. c, H70M cytochrome c₃ studied with an unmodified polished gold electrode. Potentials are expressed versus Ag/AgCl (saturated NaCl) reference electrode.
Site-directed Mutagenesis on Tetraheme Cytochrome $c_3$
